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(54) Title: ALLELE-SPECIFIC PRIMER EXTENSION ASSAY

(57) Abstract: The present invention provides methods of allele-specific primer extension useful for detecting mutations and genetic variations. In particular the invention provides a method of detecting a base at a pre-determined position in a nucleic acid molecule, said method comprising: performing primer extension reactions using base-specific detection primers, each primer being specific for a particular base at said predetermined position, and comparing said primer extensions to determine which base is present at said position, wherein said primer extension reactions are performed using labelled nucleotides and wherein a nucleotide-degrading enzyme is present during the primer extension reaction.

ALLELE-SPECIFIC PRIMER EXTENSION ASSAY

The present invention relates to an improved assay for detecting mutations and genetic variations based upon the use of allele-specific primers.

Analysis of single nucleotide polymorphisms (SNPs) is useful in applications including mapping, linkage studies and pharmacogenomics. Consequently, a number of different techniques have been proposed to scan these sequence variations in a high-throughput fashion. Many of these methods originate from hybridization techniques to discriminate between allelic variants. High-throughput hybridization of allele-specific oligonucleotides can be performed on microarray chips (Wang et al. (1998) Science 280:1077), microarray gels (Yershov et al. (1996) Proc. Natl. Acad. Sci. USA 93:4913), or by using allele-specific probes (molecular beacons) in the polymerase chain reaction (PCR) (Tyagi et al. (1998) Nat. Biotechnol. 16:49). Other technologies which have been shown to be useful for SNP genotyping are minisequencing (Pastinen et al. (1997) Genome Res. 7:606), mass spectrometry (Laken et al. (1998) Nat. Biotechnol. 16:1352) and pyrosequencing (Ahmadian et al. (2000) Anal. Biochem. 280:103), the latter relying on incorporation of nucleotides by DNA polymerase with an enzymatic cascade converting the released pyrophosphate (PPi) into detectable light.

The use of pairs of allele-specific primers with alternative bases at the 3' end has been used to identify single base variations. Higgins et al. (1997) Biotechniques 23:710; Newton et al. (1989) Lancet 2:1481; Goergen et al. (1994) J. Med. Virol. 43:97; and Newton et al. (1989) Nucleic Acids Res. 17:2503. This method exploits the difference in primer extension efficiency by a DNA polymerase of a matched over a

mismatched primer 3'-end. Generally, a sample is divided into two extension reaction mixtures that contain the same reagents except for the primers, which differ at the 3'-end. The alternating primer is designed to match one allele perfectly but mismatch the other allele at the 3'-end. Because the polymerase differs in extension efficiency for matched versus mismatched 3'-ends, the allele-specific extension reaction thus provides information on the presence or absence of one allele.

The foregoing method of identifying single base variations using allele-specific primers with varying 3'-ends suffers from certain deficiencies. In particular, certain mismatches, such as G:T and C:A, are poorly discriminated by the DNA polymerase, leading to false positive signals (Day et al. (1999) Nucleic Acids Res. 27:1810). In these cases, DNA polymerase extends the mismatched primer-templates in the presence of nucleotides although, as the present inventors have shown, with slower reaction kinetics as compared to extension of the matched configuration. However, the kinetic difference is usually not distinguishable in end point analysis, such as allele-specific PCR.

The present invention solves the deficiencies of the prior art by providing a method of allele-specific extension that allows accurate discrimination between matched and mismatched configurations. The present methods are useful for high throughput SNP analysis.

The present invention provides methods of allele-specific primer extension useful for detecting mutations and genomic variations. In one embodiment, a nucleotide degrading enzyme, preferably apyrase, is included in the allele-specific primer reaction. In this method, nucleotides are degraded before extension in reactions having slow kinetics due to mismatches, but not in reactions having fast kinetics due to matches of primer and allelic target.

In another embodiment, the primers for the allele-
specific primer extension reactions are designed such
that the 3'-end base is complementary to the target, the
penultimate (3'-1 end) base is allele-specific, and the
5   base two positions from the 3'-end (3'-2 end) is non-
complementary to (e.g. the same as) the target. When
one mismatch is present (2 bases from the 3-end) the
primer target duplex is stable and extension occurs.
However, when two mismatches are present (at the 3'-1
10   and 3'-2 positions), duplex stability is disrupted and
no detectable extension occurs.

Fig. 1 depicts the results of allele-specific
extension for the three variants of the single
nucleotide polymorphisms codon 72 (p53) and wiaf 1764.
15   The SNP status, which was determined separately for each
sample, and the extension primers are shown at the top
of the Figure. Fig. 1a shows the raw data obtained
using the luminometric assay without apyrase (top panel)
and with apyrase (lower panel). The arrows point out
20   the signal of pyrophosphate (0.02 :M) which was added to
the reaction mixture prior to the nucleotide addition in
all samples to serve as a positive control as well as
for peak calibration. Fig. 1B shows the extension
results of these samples using fluorescently labelled
25   nucleotides spotted on a glass slide, with apyrase
(lower panel) and without apyrase (top panel).

Fig. 2 is a schematic depiction of a method of
apyrase mediated allele specific extension using
barcodes as tags on the 5'-ends of allele-specific
30   primers.

Fig. 3 is a schematic depiction of a method of
allele-specific extension in solution followed by
hybridization to a DNA microarray.

Fig. 4 is a schematic depiction of an apyrase
35   mediated allele specific reaction in which Taq Man
probes are used to measure primer extension.

Fig. 5 depicts raw data of an apyrase mediated

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allele specific reaction utilizing a microarray format.

Fig. 6 depicts bioluminometric analysis of a SNP by double allele-specific primer extension on double stranded DNA.

Fig. 7 depicts allele specific extension using a primer having an introduced mismatch.

Fig. 8 is a schematic depiction of a method of detecting unknown mutations using partially overlapping extension primers.

Fig. 9 is a diagram showing the average intensities measured in an allele specific extension reaction.

The present invention relates to methods for detecting bases in a nucleic acid target. The present methods are useful for detecting mutations and genomic variations, and particularly single nucleotide polymorphisms (SNPs), and may be used with single stranded or double stranded DNA targets.

In one embodiment, the present invention provides a method of detecting a base at a predetermined position in a DNA molecule. The method utilizes DNA polymerase catalyzed primer extension. Pairs of primers are designed that are specific for (i.e. complementary to) an allele of interest, but that differ at the 3'-terminus, which position corresponds to the polymorphic nucleotide. In one primer, the 3'-terminus is complementary to the non-mutated nucleotide; in the other primer, the 3'-terminus is complementary to the mutated nucleotide. The primers are used in separate extension reactions of the same sample. Depending upon whether the mutation is present, the 3'-terminus of the primer will be a match or mismatch for the target. The DNA polymerase discriminates between a match and mismatch, and exhibits faster reaction kinetics when the 3'-terminus of the primer matches the template. Thus measurement of the difference in primer extension efficiency by the DNA polymerase of a matched over a mismatched 3'-terminus allows the determination of a

non-mutated versus mutated target sequence. In
accordance with the present invention it has been found
that the addition of a nucleotide degrading enzyme in
the extension reaction minimizes the extension of
5   mismatched primer configurations by removal of
nucleotides before incorporation but allows extension
when reaction kinetics are fast, i.e. in a matched
configuration. The present invention thus reduces or
eliminates false positive results seen in prior art
10   methods.

        In this aspect the present invention thus provides
a method for detecting an allele-specific base at a
predetermined position in a target nucleic acid molecule
comprising providing a first and a second hybridization
15   mixture, said first hybridization mixture comprising
said target nucleic acid molecule, and a primer that
hybridizes to a region of said target nucleic acid
molecule and that has a 3'-terminus that is
complementary to a non-mutated base at said
20   predetermined position, said second hybridization
mixture comprising said target nucleic acid molecule,
and a primer that hybridizes to a region of said target
nucleic acid molecule and that has a 3'-terminus that is
complementary to a mutated base at said predetermined
25   position; adding a primer extension reaction mixture to
each of said first and second hybridization mixtures,
said primer extension reaction mixture comprising a DNA
polymerase, nucleotides, and a nucleotide degrading
enzyme; and determining primer extension efficiency in
30   each of said first and second mixtures, whereby greater
efficiency in the mixture comprising the primer having a
3'-terminus that is complementary to the mutated base is
indicative of the presence of the mutated base in the
target nucleic acid, and whereby greater efficiency in
35   the mixture comprising the primer having a 3'-terminus
that is complementary to the non-mutated base is
indicative of the presence of the non-mutated base in

the target nucleic acid.

In accordance with the present invention, samples
may be prepared, primers synthesized, and primer-
extension reactions conducted by methods known in the
5   art, and disclosed for example by Higgins et al. (1997)
Biotechniques 23:710; Newton et al. (1989) Lancet
2:1481; Goergen et al. (1994) J. Med. Virol. 43:97; and
Newton et al. (1989) Nucleic Acids Res. 17:2503, the
disclosures of which are incorporated by reference. SNP
10   sites may be amplified prior to analysis, for example by
PCR, including nested and multiplex PCR. SNP containing
templates may be immobilized. This publication also
describes how the nucleotide-degrading enzyme may be
used.

15   The nucleotide degrading enzyme is preferably added
after primer hybridization and preferably with the
extension reaction mixture. For PCR reactions, a
thermostable nucleotide degrading enzyme is used. As
explained in detail in the Applicant's earlier
20   application No. WO98/28440 relating to the use of
nucleotide-degrading enzymes in pyrophosphate-detection
based sequencing methods, any nucleotide-degrading
enzyme may be used, capable of non-specifically
degrading nucleotides. This publication also describes
25   how the nucleotide-degrading enzyme may be used. Thus,
the term "nucleotide-degrading enzyme" as used herein
includes all enzymes capable of non-specifically
degrading nucleotides, including at least nucleoside
triphosphates (NTPs), but optionally also di- and mono-
30   phosphates, and any mixture or combination of such
enzymes, provided that a nucleoside triphosphatase or
other NTP degrading activity is present. Although
nucleotide-degrading enzymes having a phosphatase
activity may conveniently be used according to the
35   invention, any enzyme having any nucleotide or
nucleoside degrading activity may be used, e.g. enzymes
which cleave nucleotides at positions other than at the

phosphate group, for example at the base or sugar
residues. Thus, a nucleoside triphosphate degrading
enzyme activity is essential for the invention.
Nucleoside di- and/or mono-phosphate degrading enzymes
or enzyme activities are optional and may be used in
combination with a nucleoside tri-phosphate degrading
enzyme activity. Suitable such enzymes include most
notably apyrase which is both a nucleoside diphosphatase
and triphosphatase, catalysing the reactions NTP → NMP +
2Pi and NTP → NDP + Pi (where NTP is a nucleoside
triphosphate, NDP is a nucleoside diphospate, NMP is a
nucleotide monophosphate and Pi is phosphate). Apyrase
may be obtained from Sigma Chemical Company. Other
suitable nucleotide triphosphate degrading enzymes
include Pig Pancreas nucleoside triphosphate
diphosphohydrolase (Le Bel et al., 1980, J. Biol. Chem.;
255, 1227-1233). Further enzymes are described in the
literature.

Different combinations of nucleoside tri-, di- or
monophosphatases may be used. Such enzymes are
described in the literature and different enzymes may
have different characteristics for deoxynucleotide
degradation, eg. different Km, different efficiencies
for a different nucleotides etc. Thus, different
combinations of nucleotide degrading enzymes may be
used, to increase the efficiency of the nucleotide
degradation step in any given system. For example, in
some cases, there may be a problem with contamination
with kinases which may convert any nucleoside
diphosphates remaining to nucleoside triphosphates, when
a further nucleoside triphosphate is added. In such a
case, it may be advantageous to include a nucleoside
disphosphatase to degrade the nucleoside diphosphates.
Advantageously all nucleotides may be degraded to
nucleosides by the combined action of nucleoside tri-,
di- and monophosphatases.

Generally speaking, the nucleotide-degrading enzyme

is selected to have kinetic characteristics relative to
the polymerase such that nucleotides are first
efficiently incorporated by the polymerase, and then any
non-incorporated nucleotides are degraded. Thus, for
example, if desired the K_m of the nucleotide-degrading
enzyme may be higher than that of the polymerase such
that nucleotides which are not incorporated by the
polymerase are degraded. Thus, the nucleotide-degrading
enzyme or enzymes are simply included in the polymerase
reaction mix. The amount of nucleotide-degrading enzyme
to be used, may readily be determined for each
particular system, depending on the reactants selected,
reaction conditions etc.

     As mentioned above, the nucleotide-degrading
enzyme(s) may be included during the polymerase reaction
step. This may be achieved simply by adding the
enzyme(s) to the polymerase reaction mixture prior to or
simultaneously with the polymerase reaction (i.e. the
chain extension or nucleotide incorporation), e.g. prior
to or simultaneously with, the addition of the
polymerase and/or nucleotides to the sample/primer.

     In one embodiment, the nucleotide-degrading
enzyme(s) may simply be included in solution in a
reaction mix for the polymerase reaction, which may be
initiated by addition of the polymerase or
nucleotide(s).

     In a preferred embodiment of the present method,
the nucleotide degrading enzyme is apyrase, which is
commercially available, for example from Sigma Chemical
Co., St. Louis, Mo. USA. Those of ordinary skill in the
art can determine a suitable amount of apyrase or other
nucleotide degrading enzyme to degrade unincorporated
nucleotides in extension reactions having slow kinetics
due to a mismatch at the 3'-terminus of the primer. For
example, a typical 50 :l extension reaction mixture may
contain for 1 to 15 mU, and preferably from 5 to 10 mU,
of apyrase.

In the present invention, primer extension can be measured, and thus the ratio of extension using the primer having a matched 3'-terminus and extension using the primer having an unmatched 3'-terminus determined, by methods known in the art. If two primers are used in each reaction, PCR products can be generated and measured. Other methods of measuring primer extension include mass spectroscopy (Higgins et al. (1997) Biotechniques 23:710), luminometric assays, in which incorporation of nucleotides is monitored in real-time using an enzymatic cascade, (Nyren et al. (1997) Anal. Biochem. 244:367), fluorescent assays using dye-labelled nucleotides, or pyrosequencing as described by Ronaghi et al. (1998) Science 281:363, and Ahmadian et al. (2000) Anal. Biochem. 280:103.

It has been found that the method of the invention may advantageously be performed using labelled nucleotides in the primer extension reactions to provide a convenient way of monitoring (e.g. detecting or measuring) the primer extension reactions; it may be detected whether or not incorporation has taken place by detecting the label carried on the nucleotide, or the relative or respective rates (or efficiencies) of nucleotide incorporation.

In a preferred aspect, the invention thus provides a method of detecting a base at a pre-determined position in a nucleic acid molecule, said method comprising:

performing primer extension reactions using base-specific detection primers, each primer being specific for a particular base at said predetermined position, and comparing said primer extensions to determine which base is present at said position, wherein said primer extension reactions are performed using labelled nucleotides and wherein a nucleotide-degrading enzyme is present during the primer extension reaction.

As explained above, this method of the invention

may be used to detect a base change at a particular pre-
determined position, by determining which base is
present at that position. The method may thus be used
to detect mutations, or genetic variation (e.g. allelic
variation) based on base changes. The base detected may
thus be allele-specific. The method has particular
utility in the detection of single base changes or
single nucleotide polymorphisms (SNPs). The primers
used may thus be allele-specific. The method may be
used to detect base changes (e.g. polymorphisms or
mutations) which are known or unknown. Thus known or
unknown base changes may be identified.

As discussed above, allele-specific assays based on
the use of allele-specific primers are well known in the
art and widely described in the literature. Any such
"allele-specific" primers may be used as the "base-
specific" primer in the method of the invention. Thus,
the base-specific primer is designed to be specific for,
or selective for, a particular base, for example a base
the presence or absence of which it is desired to
detect. Such a primer will only be extended (e.g.
detectably extended) or will have a greater extension
efficiency (or higher rate of extension) in the presence
of that base in the target (i.e. pre-determined)
position in the target nucleic acid molecule which acts
as template in the primer extension reaction, and will
not be extended (or will be extended at a lower rate or
with reduced efficiency) in the absence of that base.
Such primers therefore "match" or "mismatch" the
particular base for which they are selective or
specific.

Most conveniently, as discussed above, such base-
specific, or allele-specific primers may be designed to
comprise a "match" or "mismatch" for the target base at
their 3' end (i.e. at the 3' terminus). Thus 3' base
(i.e. the base at the 3' terminus) of the primer is
designed either to match (i.e. to be complementary) to a

particular base (e.g. a particular allele, or mutation)
or mismatch it (i.e. be non-complementary). As
explained above, in the context of a mutation, in one
primer the 3' base may be complementary to the non-
mutated (e.g. wild-type) base and in a second primer
i.e. may be complementary to a mutated base (e.g. the
mutation it is desired to detect).

Thus, in the context of a known mutation or
biallelic variation (e.g. SNP), for example, two primers
may be used (so called allele-specific primers) the
first "matched" to the one base which may occur, and the
second "matched" to the other base which may occur.
Where more than one base may occur, more than two base-
specific primers may be used, each specific for (or
"matched to") each of the bases which may occur.

The method of the invention thus comprises the use
of at least two (or two or more) base-specific primers,
e.g. 2, 3 or 4 primers. As discussed further below, the
method of the invention may be adapted to the detection
of unknown mutations, simply by providing primers
specific for each of the 4 bases (A, T, C or G) which
may occur at a particular, (i.e. pre-determined)
position. Thus, each position in a target sequence may
be "scanned" for mutations.

Although, as mentioned above, the use of base-
specific primers which differ at their 3' terminus
represents one way of performing the method, the method
of the invention is not so limited, and alternative
primer designs are also possible, as again is well known
and widely described in the art. Thus, allele-specific
(or base specific) primers according to the present
invention may comprise a match (or mismatch) for the
target base at other positions, for example at the 3'-1
position of the primer (i.e. the penultimate base of the
primer). Thus the "allele-specific" (or "base-
specific") base in the primer need not be the 3' base,
but it may be present at other positions e.g. the -1 or

-2 (ante-penultimate) position. In addition, the primer
may also comprise other modifications (e.g. other
mismatches) at other positions to aid or improve "base-
specify" (i.e. discrimination between the "matched" and
"mismatched" situation). Again, such principles of
primer design are also discussed in the literature. A
further aspect of the present invention is based on
modified primer design and is discussed further below.

The primer extension reactions may be performed as
described above. For example the target nucleic acid
molecule may be contacted with each said base-specific
primer in conditions under which hybridisation (or
annealing) of the primers, and subsequently primer
extension, may occur. Conveniently, "hybridization
mixtures" comprising template (i.e. target nucleic
molecule) and primer may be prepared (i.e. 1 to 4
hybridisation mixtures depending on the number of
primers used). Polymerase and nucleotides for the
performance of the primer extension reaction may then be
added. As explained above, the nucleotide degrading
enzyme may be included in the primer extension reaction
mixture, or added separately.

The primer extension reactions performed using the
different base-specific primers are then compared. More
particularly, in this comparison step, the rates of
extension may be compared (e.g. the respective or
relative rates). Thus, efficiencies of the primer
extension reactions may be compared.

The respective rates, or efficiencies, of the
primer extension reactions may be determined for each of
the different primers and compared. A greater
efficiency, or higher extension rate, is indicative of
the presence of the base for which the primer is
specific (i.e. "matched"). Thus, if a detection (i.e.
base-specific) primer matches the template (i.e. the
target base) a high extension rate (or efficiency) will
be observed. If the primer is not a matched primer, the

extension rate (or efficiency) will be lower. The
difference in primer extension rate (or efficiency) over
a matched or mismatched primer can be used for
discrimination between bases, and thus to determine
5   which base is present at said pre-determined position.

It has been found that the use of labelled
nucleotides, represent a particularly advantageous way
of detecting, and comparing, the primer extension
reactions. Thus, the nucleotides used for incorporation
10   in the primer extension reaction may carry a label,
preferably a detectable label, which may be used to
detect whether or not the nucleotide has been
incorporated, or to follow or monitor the primer
extension reaction to detect the rate or efficiency of
15   nucleotide incorporation. Such a label may be any label
giving or leading to a detectable signal (which may be
directly or indirectly detectable, for example a moiety
which takes part in a reaction (e.g. an enzymic or
antibody-based reaction) which generates a detectable
20   signal). Directly detectable labels are preferred, and
especially labels based on dyes which may be
spectrophotometrically or fluorescently detectable.
Fluorescent labels are especially preferred.

Labelled nucleotides for use in sequencing are well
25   known in the art and widely described in the literature
and any of these may be used. WO 00/53812, for example,
describes, *inter alia*, methods of sequencing based on
dye-labelled (particularly fluorescently labelled)
nucleotides, which may be used according to the present
30   invention. See also US-A-6,087,095.

In preferred embodiments, the label, for example
the dye (e.g. fluorophore), may be attached to the
nucleotide via a cleavable linker which can be cleaved
to remove the label from the nucleotide when desired.
35   Cleavage may be accomplished, for example, by chemical
reaction (e.g. acid or base treatment) or by oxidation
or reduction of the linkage or by light treatment, or

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enzymically, depending on the nature of the linkage.
Such cleavable linkages are described in detail in WO
00/53812. A preferred example is a disulphide linkage
which may be cleaved by reduction, e.g. with a reducing
agent such as dithiothreitol. Dyes, for example
fluorophores, e.g. cyanine fluorophores (e.g. cyanine 3
or cyanine 5 fluorophores) may be conjugated via
disulphide bonds to a nucleotide as described in WO
00/53812. Advantageously the label may be attached to
the 3' moiety of the deoxyribose group of a nucleotide,
such that cleavage and removal of the label group from
the nucleotide generates a 3' OH group. Modified
labelled nucleotides known as "false terminators" where
a blocking group may be removed from the modified
nucleotide are also described in EP-A-0745688 and US-A-
5,302,509.

The present method may be performed in a solid
phase microarray format, for example on a chip, whereby
samples are extended in solution followed by
hybridization to a microarray. (Fig. 3).
Alternatively, the method may be performed in a
microarray format in which the primers or samples
(templates) are immobilised. Allele-specific primer
extension on microarrays is known in the art and
described for example by Pastinen et al. (2000) Genome
Research 10:1031. The method may also be performed a
liquid phase assay using barcodes as tags on the 5'-ends
of allele-specific primers as described by Fan et al.
(2000) Genome Res. 10:853. By using barcodes, a liquid-
phase multiplex apyrase mediated allele specific
extension of a set of SNPs may be performed in a single
tube (if the barcodes on the matched and mismatched
primers are different) or in two tubes (if the barcodes
on the matched and mismatched primers are identical).
After primer extension, the extension products can be
hybridized to barcodes on the chip. A modular probe as
described by O'Meara et al. (1998) Anal. Biochem.

255:195 and O'Meara et al. (1998) J. Clin. Microbiol.
36:2454 may be utilized to improve the hybridization
efficiency. The modular probe hybridizes to its
complementary segment on the immobilized oligonucleotide
5   and improves the hybridization of a barcode that is
immediately downstream.

        The present method may also be performed using "Taq
Man" probes, which are probes labelled with a donor-
acceptor dye pair that functions via fluorescence
10   resonance transfer energy (FRET) as described by Livak
et al. (1995) PCR Methods Appl. 4:357. When Taq Man
probes are hybridized to a target, the fluorescence of
the 5'-donor fluorophore is quenched by the 3'-acceptor.
When the hybridized probe is degraded, the 5'-donor dye
15   dissociates from the 3'-quencher, leading to an increase
in donor fluorescence.

        After PCR amplification, a Taq Man probe is used
which hybridizes 15 to 20 bases downstream of a 3'-end
allele-specific primer. Apyrase-mediated allele
20   specific extension is then performed. In the case of a
matched primer - template, DNA polymerase extends the
primer and degrades the Taq Man probe by its 5'-nuclease
activity, thus leading to increased donor fluorescence.
In the mismatched case, apyrase degrades the nucleotides
25   and the fluorescence level remains unchanged. Such an
assay is depicted in Figure 4.

        Extension products may also be distinguished by
mass difference or by the use of double-stranded-
specific intercalating dyes.
30   In another embodiment of the present invention,
allele specific primer extension with a nucleotide
degrading enzyme is performed using a double stranded
DNA template. If double stranded DNA is generated by
PCR, the excess of primers, nucleotides and other
35   reagents must first be removed by methods known in the
art such as treatment with alkaline phosphatase and
exonuclease I. Two pairs of allele-specific primers are

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used. One pair is complementary to the forward strand
and one to the reverse strand. The primer pairs differ
in their 3'-ends as described above. Allele-specific
extension is performed on both strands of the double-
5   stranded DNA.

As mentioned above, in one embodiment, the present
invention provides a method of detecting a mutation in a
DNA molecule. In this method, the mutation is not pre-
defined, as in a SNP (i.e. it is unknown). The method
10   utilizes DNA polymerase catalyzed primer extension. Four
primers are designed that each comprise a region that is
specific for (i.e., complementary to) a target nucleic
acid of interest or to a region interest in a target
nucleic acid, e.g. a particular stretch of nucleotides,
15   but that differ (e.g. at the 3'-terminus) such that
primer has a different nucleotide base (A, C, G or T) at
a "base specific" (or "allele-specific" position), for
example at the 3'-terminus. As mentioned above,
alternatively the "allele-specific" base or different
20   nucleotide may be present at another position, e.g. the
3'-1 position. The primers are used in separate
extension reactions of the same sample of target nucleic
acid. Depending upon which base is present in the
target nucleic acid at the position corresponding to the
25   3'-terminus of the primer (or any other "allele-
specific" position), only one of the primers will match
the target. The DNA polymerase discriminates between a
match and mismatch, and exhibits faster reaction
kinetics when the 3'-terminus allele-specific base of
30   the primer matches the template. Thus measurement of
the difference in primer extension efficiency by the DNA
polymerase of the matched over the three mismatched 3'-
termini allows determination of the base at the position
in the target nucleic acid that corresponds to the 3'-
35   terminus of the primer (or analogously, at any other
"base or allele-specific" position). Accordingly, if a
previously unknown mutation is present in the target

nucleic acid, the mutation is detected by virtue of one
of the four primers "matching" the mutated target, and
that primer exhibiting the greatest extension efficiency
(or highest extension rate). Thus, the identity of the

5   target base may be determined by determining which
primer is extended (i.e. which base is incorporated)
with the greatest rate or efficiency (or highest
extension rate). In accordance with the present
invention it has been found that the addition of a

10   nucleotide degrading enzyme in the extension reaction
minimizes the extension of mismatched primer
configurations by removal of nucleotides before
incorporation, but allows extension when reaction
kinetics are fast, i.e., in a matched configuration,

15   thereby reducing or eliminating false positive results.
        Samples may be prepared, primers synthesized, and
primer-extension reactions conducted by methods known in
the art as described hereinabove. Primers may be
immobilized. Samples of target nucleic acids may be

20   amplified prior to analysis, for example by PCR,
including nested and multiplex PCR. Target nucleic
acids may be immobilized.
        In a preferred embodiment of such a mutation-
detection method, each position in a target nucleic acid

25   (or in a selected or target region) may be evaluated by
using overlapping sets of extension primers. Thus, each
position in a target region may be scanned for mutations
using this method of the invention, simply by
appropriately selecting or designing a detection primer

30   such that the base at the selected "allele-specific"
position in the primer (e.g. the 3'-terminus)
corresponds to, or is complementary to the base to be
scanned, i.e. the target base at the desired position.
The primers may be spotted onto a surface to provide an

35   array, followed by hybridization to target nucleic acid,
extension, measurement of extension, and comparison of
extensions.

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In another embodiment of the present invention, allele specific extension is performed using a pair of primers in which the 3'-end base is complementary to the target, the penultimate (3'-1 end) base is allele-specific (mutated or nonmutated) and the base two positions from the 3'-end (3'-2 end) is non-complementary to (for example the same as) the target. When the allele-specific base in the primer matches the target template, i.e. one mismatch is present (2 bases from the 3'-end), the primer-target duplex is stable and extension occurs. When the allele-specific base does not match the target, i.e. two mismatches are present (at the 3'-1 and 3'-2 positions), duplex stability is disrupted and no detectable extension occurs. The assay may be performed in all the embodiments as described above, in the presence or absence of a nucleotide degrading enzyme. An example of the method is depicted in Fig. 7.

All references cited herein are incorporated herein in their entirety.

The following non-limiting examples serve to further illustrate the present invention.

Example 1
Apyrase Mediated Allele Specific Extension
Experimental protocol

Samples, PCR and single strand preparation

Human genomic DNA was extracted from twenty-four unrelated individuals. Two duplex PCRs were performed to amplify four SNPs. The SNPs were wiaf1764 (A/C) on chromosome 9q, codon 72 (C/G) on the p53 gene (Ahmadian et al. (2000) Anal. Biochem. 280:103) nucleotide position 677 (C/T) on the MTHFR gene (Goyette et al. (1998) Mamm. Genome 9:652, erratum in (1999) Mamm. Genome 10:204) and nucleotide position 196 (A/G) on the GPIIIa gene (Newman et al. (1989) J. Clin. Invst.

83:1778). The outer duplex PCR for wiaf 1764 and p53
gene (94°C 1 min, 50°C 40s and 72°C 2 min for 35 cycles)
was followed by specific inner PCRs (94°C 1 min, 50°C 40s
and 72°C 1 min for 35 cycles) generating ~80 bp
5   fragments for each SNP (Table 1). The outer duplex PCR
condition for MTHFR and GPIIIa genes was 95°C 30s, 60°C 1
min and 72°C 1 min. This was followed by individual
inner PCRs (95°C 1 min, 66°C 50 sec and 72°C 2 min)
(Table 1). The outer and inner amplification mixtures
10   comprised of 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM
KCl, 0.1% (v/v) Tween 20, 0.2 mM dNTPs, 0.1 μM of each
primer and 1 unit of AmpliTaq DNA polymerase (Perkin-
Elmer, Norwalk, Connecticut, USA) in a total volume of
50 μl. Five microliters of total human DNA (1 ng/μl)
15   were used as outer PCR template. In the inner PCR, one
of the primers in the respective set was biotinylated at
the 5'-end to allow immobilization. 40 μl biotinylated
inner PCR products were immobilized onto streptavidin-
coated super paramagnetic beads (Dynabeads M280; Dynal,
20   Oslo, Norway). Single-stranded DNA was obtained by
incubating the immobilized PCR product in 12 μl 0.1 M
NaOH for 5 min. The immobilized strand was then used
for hybridization to extension primers (Table1).

25   *Apyrase mediated allele-specific extension using a*
bioluminometric assay
        The immobilized strand was resuspended in 32 μl H_2O
and 4 μl annealing buffer (100 mM Tris-acetate pH 7.75,
20 mM Mg-acetate). The single stranded DNA was divided
30   into two parallel reactions in a microtiter-plate (18
μl/well) and 0.1 μM (final concentration) of primers
were added to the single stranded templates in a total
volume of 20 μl. Allele discrimination between the
allelic variants was investigated by the use of Klenow
35   DNA polymerase using two separate SNP primers that only
differed in the 3'-end position (Table 1).
Hybridization was performed by incubation at 72°C for 5

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min and then cooling to room temperature. The content
of each well was then further divided into two separate
reactions for comparison of extension analysis with and
without apyrase. Extension and real-time luminometric
5   monitoring was performed at 25°C in a Luc96
pyrosequencer instrument (Pyrosequencing, Uppsala,
Sweden). An extension reaction mixture was added to the
single stranded DNA (10 μl) with annealed primer (the
substrate) to a final volume of 50 μl. The extension
10   reaction mixture contained 10 U exonuclease-deficient
(exo-) Klenow DNA polymerase (Amersham Pharmacia
Biotech, Uppsala, Sweden), 4 μg purified luciferase/ml
(BioThema, Dalarö, Sweden), 15 mU recombinant ATP
sulfurylase, 0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA,
15   5 mM Mg-acetate, 0.1% (w/v) bovine serum albumin
(BioThema), 1 mM dithiothreitol, 10 μM adenosine 5'-
phosphosulfate (APS), 0.4 mg polyvinylpyrrolidone /ml
(360 000), 100 μg D-luciferin/ml (BioThema) and 8 mU of
apyrase (Sigma Chemical Co., St. Louis, MO, USA) when
20   applicable. Prior to nucleotide addition and measuring
of emitted light, pyrophosphate (PPi) was added to the
reaction mixture (0.02 μM). The PPi served as a
positive control for the reaction mixture as well as
peak calibration. All the four nucleotides (Amersham
25   Pharmacia Biotech, Uppsala, Sweden) were mixed and were
dispensed to the extension mixture (1.4 μM, final
concentration). The emitted light was detected in real-
time.

30   *Apyrase mediated allele-specific extension using
fluorescent labeled nucleotide*

        The single stranded templates were prepared using
magnetic beads as outlined above. The immobilized
strand was resuspended in 100 μl H$_2$O and 12 μl annealing
35   buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-acetate).
The single stranded DNA was divided into two parallel
reactions in a microtiter-plate (56 μl/well) and 0.1 μM

(final concentration) of primers (Table 1) were added to
the single stranded templates in a total volume of 60
μl. Hybridization was performed by incubation at 72°C
for 5 min and then cooling to room temperature. The
reaction mixture was then further divided into two
separate reactions (30 μl) for direct comparison of the
extensions with and without apyrase. The extension
mixture contained 20 U exonuclease-deficient (exo-)
Klenow DNA polymerase (Amersham Pharmacia Biotech),
Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM Mg-acetate,
0.1% (w/v) bovine serum albumin (BioThema),1 mM
dithiothreitol and optionally 8 mU of apyrase (Sigma
Chemical Co.) in a total volume of 70 μl. The extension
mixture also contained nucleotides, dATP, dGTP, dTTP and
dCTP (1.4 μM final concentration), where dCTP was
labeled with Cy5 or Cy3 (Amersham Pharmacia Biotech).
The reaction was carried out for 15 min at room
temperature. The resulting extension products were
washed twice with Tris-EDTA and the non-biotinylated
extension products were isolated by adding 25 μl 0.1 M
NaOH. The eluted strand was neutralized by 12.5 μl 0.2
M HCl and was printed on a glass slide by the GMS 417
Arrayer (Genetic MicroSystem, USA) and then was scanned
using GMS 418 Scanner (Genetic MicroSystem, USA). The
obtained results were analyzed using GenePix2.0 software
(Axon Instruments, USA).

*Apyrase mediated allele-specific extension using
fluorescent labeled nucleotide evaluated in a microarray
format*

        After amplification, the amplicons of wiaf 1764
were immobilized onto streptavidin coated beads as
outlined above. The biotinylated inner primer was
primer 3 and not primer 4 (Table 1) with the reason that
for this assay the eluted strand is the extension
substrate, thus to have the same match and mismatch
configurations, the biotinylated inner primer was

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changed. The immobilized PCR product of wiaf 1764 was
incubated in 20 μl 0.1 M NaOH for 5 min. The eluted
strand was neutralized by 10 μl 0.2 M HCl and 4 μl
annealing buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-
5   acetate) was added. The eluted and neutralized single
stranded DNA was divided into two parallel reactions in
a microtiter-plate (17 μl/well) and extension primers
(0.1 μM) were added. Hybridization was performed by
incubation at 72°C for 5 min and then cooling to room
10   temperature. Here, the extension primers were modified
to have amino groups at the 5'-end to allow covalent
binding to pre-activated Silyated Slides (Cel Associates
Inc, Texas, USA). In order to improve hybridization and
extension the primers were extended with a 15-mer spacer
15   (T_{15}) in the 5'-end (Table 1). One microliter of the
primer-template hybrid was manually spotted on Silyated
Slides (Cel Associates Inc) and the covalent coupling
was performed in a humid chamber at 37°C for 16h. After
coupling, 1 mU apyrase (1 μl) was added to each spot. A
20   polymerization mixture was prepared and 4 μl was added
to the spots immediately after addition of apyrase. The
polymerization mixture contained Tris-acetate (pH 7.75),
0.5 mM EDTA, 5 mM Mg-acetate, 0.1% (w/v) bovine serum
albumin (BioThema), 1 mM dithiothreitol, 0.08 μM (final
25   concentration) dCTP labeled with Cy3 (Amersham Pharmacia
Biotech) and 1 U exonuclease-deficient (exo-) Klenow DNA
polymerase (Amersham Pharmacia Biotech). Polymerization
was allowed to proceed for 15 min and the microarray
slide was washed briefly with water and then scanned
30   using GMS 418 Scanner (Genetic MicroSystem, USA). The
data was analyzed by using GenePix2.0 software (Axon
Instruments, USA).

Pyrosequencing

35   Single stranded DNA with annealed sequence primer
(the substrate) (Table 1) were used for pyrosequencing.
Real-time pyrosequencing was performed at 28°C in a

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total volume of 50 μl in an automated 96-well
PyroSequencer using PSQ™ SNP 96 enzymes and substrates
(Pyrosequencing AB, Uppsala, Sweden).

5   **Results**

        Two different approaches of apyrase mediated
allele-specific extensions were investigated. The
assays included a luminometric assay and two assays
based on fluorescent labeled nucleotides. All the
10   results obtained with these assays were confirmed by
pyrosequencing. Four SNPs with the eight alternative
3'-end primer-template configurations were investigated
(Table 1 and Table 2). The SNPs were codon 72 of the
p53 gene (C or G), wiaf 1764 (G or T), nucleotide
15   position 677 in the MTHFR gene (C or T) and nucleotide
position 196 in the GPIIIa gene (G or A). Thus, using
two alternative allele-specific primers for each SNP,
the following mismatches were possible; G-G and C-C for
codon 72 (p53 gene), A-G and C-T for wiaf 1764, A-C and
20   G-T for polymorphic position on the MTHFR gene, T-G and
C-A for the SNP on the GPIIIa gene (Table 2).

        Figure 1A shows the results of the luminometric
assay with and without apyrase, for codon 72 of the p53
gene and wiaf 1764. When codon 72 is homozygous G
25   (sample g1) (see Table 2), the mismatch signal is as
high as the match signal but the slope of the curve
indicates slower reaction kinetics (Figure 1A top panel,
extension 2). The same was observed for wiaf 1764
homozygous T (sample g055) (see Table 2). However, when
30   apyrase was included in the allele-specific extension of
these samples (Figure 1A, lower panel) a dramatic
difference was observed. The previous high signals for
mismatch configurations, disappeared with the addition
of apyrase. The extension ratios were calculated by
35   taking the ratio of the high versus the low end-point
signals. For example, the ratio for sample g1 in the
p53 gene was 1:1 without apyrase and 13.9 with apyrase

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and for the sample g055 in wiaf 1764 the ratio was 1
without apyrase and 5 with apyrase (Table 2). This
clearly shows that addition of apyrase affects the
extension and simplifies interpretation.   In these
5   assays an extension ratio below or equal to 2 was
interpreted as a sample being heterozygous. If the
ratio was above or equal to 2.5 the SNP was scored as
homozygous with the nucleotide at the 3'-end of the
primer that produced the higher signal. Ratios between
10   2 and 2.5 were interpreted as uncertain. As shown in
Figure 1A and Table 2, in all cases with addition of
apyrase the extension signals and extension ratios
resulted in a correct genotype with the luminometric
assay as compared to the pyrosequencing data. In fact,
15   the extension ratios for all heterozygous SNPs were
between 1.1 and 1.4 and the lowest extension ratio for a
homozygous sample was 5. In contrast, without the
inclusion of apyrase, five out of eight mismatches
contributed with so high extension signals that the SNPs
20   were wrongly scored (ratios in bold). The primer-
template mismatches that were extended in these cases
were G-G, C-T, G-T, T-G and C-A. Figure 1B also shows
the raw data of similar extensions on DNA immobilized on
magnetic beads using fluorescent detection using a
25   labeled nucleotide instead of a luminometric detection
system. Thus, extension is performed by the same
polymerase and the same primers, meaning that the
discrimination behavior should be as in the luminometric
assay. The results (extension ratios) are in good
30   agreement with the luminometric assay (Table 2).
Without apyrase the same mismatches gave high
fluorescent signals leading to that the same homozygous
samples were wrongly scored as heterozygous (ratios in
bold in Table 2). However in presence of apyrase, no
35   ambiguities or discordant results were observed (Figure
1B lower panel and Table 2).

The foregoing example demonstrates that apyrase

aids in the discrimination of mismatches in allele-
specific extension.   The extensions in these cases were
performed on magnetic beads.   The use of this technology
in a microarray format was also evaluated.   The SNP wiaf

5   1764 were amplified in 3 samples (g011, 119 and g055)
and single stranded templates were obtained by
immobilization of the PCR product onto magnetic beads.
The eluted non-biotinylated strand was used in the
subsequent extension experiments.   Extension primers

10   were modified to have a free amino group in the 5'-end
as well as a 15 nucleotide long oligo dT spacer.   The
extension primers and the single strand target were
hybridized, printed and then covalently coupled to the
activated glass slides.   The extension reaction mixtures

15   were then added to microarray.   Figure 5 shows the raw-
data of the analysis without and with apyrase.   A direct
comparison of the raw data obtained for samples of wiaf
1764 using both assays shows that the results of
extension on chip are correct when apyrase is used while

20   the same homozygous sample as in the other two assays
(g055) has led to a high mismatch signal when apyrase
was not used.

       As shown above, a major advantage of using apyrase
mediated allele specific extension (AMASE) is that the

25   technique is applicable for high throughput genotyping.
The present method may also be performed using barcodes
(Fan et al. (2000) Genome Res.   10:853) as tags on the
5'-ends of allele-specific primers.   In this way a
multiplex AMASE of a set of SNPs is performed in a

30   single tube (if the barcodes on the match and mismatch
are different) or in two tubes (if the barcodes on the
match and mismatch are identical).   After performance of
AMASE, the double-stranded AMASE products are heat
separated and hybridized to barcode complementary

35   oligonucleotides on the chip (Figure 2).   To improve the
hybridization efficiency, at the hybridization step a
modular probe is utilized (O'Meara et al. (1998) J.

Clin. Microbiol. 36:2454). The modular probe can
hybridize to its complementary segment of the
immobilized oligonucleotide and improve the
hybridization of barcode that is immediately downstream.

5　　　　In conclusion, this example shows that single
nucleotide polymorphisms can rapidly be scored with
allele-specific primers in extension reactions.
Furthermore, the present method can be used in a high-
throughput format using microarrays.

10

Table 1. List of the primers.

SNP	Primer	5' 3'
wiaf1764	1	AGTGAAAACATTGAAAACACA
	2	AATGTTTTCACTGTCATAAAG
	3	TCCAATGTGTGAAAAATATATAC
	4-Biotin	AGAACACATACGTTTTACCA
	Extension1	ACTCCCTTCAGATCA
	Extension2	ACTCCCTTCAGATCC
	Amino-Extension1	TTTTTTTTTTTTTTTTATACAAC ACTCCCTTCAGATCA
	Amino-Extension2	TTTTTTTTTTTTTTTTATACAAC ACTCCCTTCAGATCC
	Seq	CATTTGTTAAGCTTTT
p53 codon 72	1	ATGCTGTCCCCGGACGA
	2	CAGGAGGGGGCTGGTG
	3-Biotin	TCCAGATGAAGCTCCCAG
	4	AGGGGCCGCCGGTGTA
	Extension1	GCTGCTGGTGCAGGGGCCACGC
	Extension2	GCTGCTGCTGCAGGGGCCACGG
	Seq	GCTGCTGGTGCAGGGGCCA
mthfr	1	CCTGACTGTCATCCCTATTGGCAG
	2	GGGACGATGGGGCAAGTGATG
	3-Biotin	GCTGACCTGAAGCACTTGAAGGAG
	4	GCCTCAAAGAAAAGCTGCGTG
	Extension1	GCTGCGTGATGATGAAATCGA
	Extension2	GCTGCGTGATGATGAAATCGG
	Seq	AAGCTGCGTGATGATGAAA
gp3a	1	GCCATAGCTCTGATTGCTGGACTTC
	2	GCCTCACTCACTGGGAACTCGATG
	3	GCTGGACTTCTCTTTGGGCTCCTG
	4-Biotin	ACAGTTATCCTTCAGCAGATTCTCCTT
	Extension1	TCTTACAGGCCCTGCCTCC
	Extension2	TCTTACAGGCCCTGCCTCT
	Seq	CCTGTCTTACAGGCCCTGCC
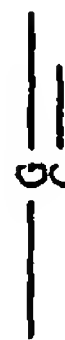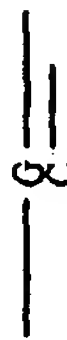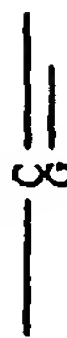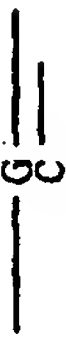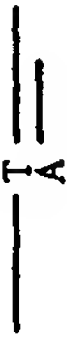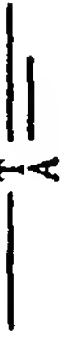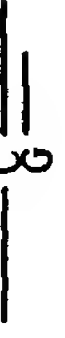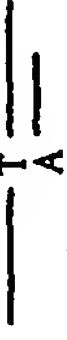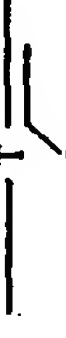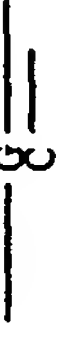
1 and 2 = primers used in the outer PCR. 3 and 4 =
45　primers used in the inner PCR. 1 and 3 are upstream
primers and 2 and 4 are downstream primers. Biotin
indicates the biotinylated primer in each set.
Extension1 and Extension2 refer to the primers used as

match and/or mismatch with the alternating base in the
3'-end. Amino-Extension refer to the primers used on
microarray. Seq = primers used for pyrosequencing.
Notice that when the Amino-Extension primers (for wiaf
1764) are used, the biotinylated inner primer was primer
3 and not primer 4

Table 2. Summary of extension results

SNP	Sample	Sequencing Result	Schematic Representation of Configurations		Allele-Specific Extension Ratio (high /low)			
			Extension 1	Extension 2	Bioluminescence		Fluorescent	
					- apyrase	+ apyrase	- apyrase	+ apyrase
p53 (G/C)	g1	G/G	G/C	G/G	1.1	13.9	1.2	4.9
	116	G/C	G/C	G/G	1.2	1.1	1.4	1.4
	123	C/C	C/C	C/G	2.6	12	2.3	10.5
wiaf1764 (G/T)	g011	G/G	G/A	G/C	8.3	8	15.5	3.9
	119	T/G	T/A	G/C	1.2	1.1	1.3	1.5
	g055	T/T	T/A	T/C	1	5	1.1	8.2
MTHFR (C/T)	1001	C/C	C/A	C/G	3.5	12	20	35
	1055	T/C	T/A	C/G	1	1.3	1.1	1.3
	1004	T/T	T/A	T/G	1.1	12	2.3	13
GPIIIa (G/A)	1267	G/G	G/C	G/T	1.1	6.5	1.1	3.5
	1001	G/A	G/C	A/T	1.2	1.4	1.4	1.1
	1055	A/A	A/C	A/T	1.4	10	1.1	7.1

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Example 2
Apyrase mediated allele-specific extension on DNA microarrays

Apyrase mediated allele-specific extension (AMASE) for genotyping on DNA microarrays is described in this report. The method involves extension of the DNA samples in solution followed by hybridization to the DNA microarray as illustrated in Figure 3.

Materials and Method

Microarray preparation

Amino linked oligonucleotide capture probes suspended at a concentration of 20μM in 3X SSC/0.01% sakrosyl were spotted using a GMS 418 arrayer (Affymetrix, USA) on silylated slides (CEL Associates, Houston, TX). Printed arrays were allowed to dry for 12 hours at room temperature followed by post processing to reduce unreacted aldehyde groups thereby minimising non-specific binding of target. Briefly the slides were washed twice in 0.2% SDS for 2 minutes, twice in dH$_2$O for 2 minutes and treated with sodium borohydride (0.75g NaBH$_4$ dissolved in 225 ml PBS and 75 ml 100% ethanol) for 5 minutes. The arrays were then washed in 0.2% SDS three times for 1 minute, rinsed in H2O and dried by centrifugation for 1 minute at 500g. Prior to use in hybridization, the arrays were prehybridized with buffer containing 5X Denharts solution, 6X SSC, 0.5% SDS and 0.1 μg/μl herring sperm DNA at 50 °C for 15 minutes followed by a brief rinse in dH$_2$O.

Oligonucleotides

The capture probes were synthesised with an amino group at the 5' end to facilitate covalent immobilisation on the glass slide. A carbon spacer was also synthesised at the 3' end to prevent any possible extension of the capture probe during hybridization,

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albeit unlikely due to the high salt conditions. The
sequences of the capture probes and the allele specific
extension primers are listed in Table 3.

5   DNA preparation
        PCR was carried out on human genomic DNA as
previously described (Ahmadian et al. (2000) Anal.
Biochem. 280:103)) to amplify 3 SNPs. The SNPs were
codon 72 (C/G) in the p53 gene, nucleotide position 677
10   (C/T) in the methylenetetrahydrofolate reductase (MTHFR)
gene and nucleotide position 196 (A/G) on the
glycoprotein IIIa (GP3a) gene. To allow for
immobilization of the PCR products on streptavidin beads
and preparation of single strand DNA, biotinylated inner
15   PCR primers were used. The biotinylated PCR-products (~
80 bp) were immobilised onto streptavidin-coated
paramagnetic beads (Dynabeads® M-280, Dynal, Oslo,
Norway) and by strand-specific elution a pure template
for extension was obtained. Briefly, 100 μl of the PCR-
20   products was captured by incubation for 15 minutes at
room temperature with 5 mg/ml of beads in 100 μl
binding/washing buffer (10 mM Tris-HCl (pH 7.5) 1 mM
EDTA, 2 M NaCl, 1 mM ß-mercaptoethanol, 0.1 % Tween®
20). After washing and removal of supernatant, the
25   strands were separated by incubation with 4 μl of 0.1 M
NaOH for 5 minutes. The alkaline supernatant with the
non-biotinylated strand was neutralised with 2.2 μl of
0.17 mM HCl and 1 μl of 100 mM Tris-Acetate pH 7.5, 20
mM MgAc$_2$.
30

Extension and hybridization
Single strand DNA:
        The single strand DNA prepared above was divided
into two aliquots and 2.5 pmoles of allele specific
35   primers were annealed by incubation at 72 °C for 5
minutes in a volume of 20 μl. The annealed primer-DNA
template was then further divided into two separate

reactions for direct comparison of extension with and
without apyrase. When multiplex extension was
performed, single strand DNA from the 3 templates was
mixed and allele specific primers were annealed.

5   Extension was performed in solution on 10 μl of the
annealed DNA (corresponds to 25 ul of each PCR product)
in a 60 μl volume containing 100 mM Tris-Acetate, 0.5 mM
EDTA and 5 mM Mg-acetate with 1.4 μM pmoles of cy5
labelled dNTPs, 2.5 μg BSA, 1.25 mM DTT, 10 Units
10   exonuclease-deficient (exo-) Klenow DNA polymerase and
optionally 8 mU apyrase. Following incubation of the
extension products at room temperature for 15 minutes,
60 μl 10X SSC/0.4% SDS was added to the extension
reactions and 100 :l was then hybridized to the
15   oligonucleotide microarray. Hybridization was performed
on the GeneTAC hybridization station (Genomic solutions,
MI, USA) at 50 °C for 20 minutes followed by washing in
2X SSC/0.1% SDS for 5 minutes proceeded by washing in
0.6X SSC for a further 5 minutes. The slides were
20   briefly rinsed in H$_2$O and dried by centrifugation at 500g
for 1 minute.

Double strand DNA:
       One hundred and sixty microlitres of PCR product
was treated with 16 Units of calf alkaline phosphatase
25   and 32 Units exonuclease I at room temperature for 30
minutes. The enzymes were inactivated by incubation at
95 °C for 12 minutes and the DNA was divided into two
aliquots and annealed to 5 pmoles of allele specific
primers (incubation at 95° C for 2 minutes followed by
30   incubation at 72 °C for 5 minutes) in a volume of 100
μl. The annealed primer-DNA template was then further
divided into two separate reactions for direct
comparison of extension with and without apyrase.
35   Extension was carried out on 50 μl of annealed double
strand DNA (corresponds to 40 μl PCR product) in a 100μl
as described above. Following incubation at RT for 15

minutes, 25 μl 20X SSC and 12 μl 10% SDS was added to
the extension mixture and 100 μl was hybridized to the
slide as described above.

5   Data analysis

The slides were scanned at optimal laser/PMT values
using the GMS 417 scanner (Affymetrix, USA) and the
features quantitated using GenePix 2.0 software (Axon
Instruments, USA). Since different
10   extension/hybridization experiments were compared, the
micorarray data was subjected to a normalization
procedure. This involved including a 66 mer
oligonucleotide control and 18 mer extension probe
(Table 3) together with the target DNA that was
15   subjected to extension and hybridization. Since the
intensity of this control should be constant from slide
to slide it was used to normalize the slides for
comparative purposes. Extension ratios were calculated
by taking the ratio of the high versus the low signal.
20

Results

Results of AMASE for simultaneous genotyping of 3
SNPs on DNA microarrays are shown in Table 4. Inclusion
of apyrase resulted in the correct genotype being called
25   in all cases. However when apyrase was omitted, SNPs
were incorrectly genotyped in 3 samples (illustrated in
boldface type) if the criteria of a ratio $\geq$ 2.5 is
required to call a homozygous genotype and $\leq$1.5 for a
heterozygous sample. Two replicates of each feature
30   were spotted which allows an estimation of the
variability of the method and the standard deviation for
each feature is shown in Table 4. The results in Table
4 are based on extension of single strand DNA while
preliminary results for genotyping of double strand DNA
35   gave ratios of 3.9$\pm$0.2 (with apyrase) versus 2.7$\pm$0.4
(without apyrase) for codon 72 of the p53 gene (sample
g1).

Table 3. Sequence of capture and extension probes

SNP	Probe	Sequence (5'-3')
P53	Capture probe Extension 1 Extension 2	TGA AGC TCC CAG AAT GCC AGA GGC TGC TCC CCC AGA GGC TGC TCC CCG
MTHFR	Capture probe Extension 1 Extension 2	CAG CCT CAA AGA AAA GCT GCG TGA TGA TGA AAT CGG GCG TGA TGA TGA AAT CGA
GPIIIa	Capture probe Extension 1 Extension 2	CTT CTC TTT GGG CTC CTG TCT TAC AGG CCC TGC CTC C TCT TAC AGG CCC TGC CTC T
Control	Capture probe Extension probe Extension template	GGT GCA CGG TCT ACG AGA CCT CCC GGG GCA CTC GCA AGG CCT TGT GGT ACT GCC TGG TAG GGT GCT TGC GAG TGC CCC GGG AGG TCT CGT AGA CCG TGC ACC

Table 4. Genotyping of SNPs on microarrays using AMASE

SNP	Sample	Sequencing Result	Allele-Specific Extension Ratio(high /low)	
			Microarray	
			- apyrase	+ apyrase
p53 (G/C)	g1	G/G	1.9±0.2 *	g1
	116	G/C	2.5±0.5*	116
	123	C/C	22.1±0.5	123
THFR (C/T)	1001	C/C	19.8±1.8	1001
	1055	T/C	nd	1055
	1004	T/T	21.3±3.7	1004
GPIIIa (G/A)	1267	G/G	2.2±0.2	1267
	1001	G/A	nd	1001
	1055	A/A	8.6±0.7	1055

*indicates that these samples were not genotyped in a multiplex format nd (not determined) indicates that these samples have yet to be tested

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Example 3
Double Stranded DNA Analysis of a SNP

Materials and Methods

5   PCR amplification of wiaf 1764 was performed as
described in the foregoing examples. In order to
perform double-strand DNA analysis of this SNP (without
strand separation by the use of beads), the excess of
primers, nucleotides and the released PPi in the PCR had
10   to be removed. For this purpose, the enzymes shrimp
alkaline phosphatase (4 U) (Roche Diagnostics) and E.
coli exonuclease I (8 U) (Amersham Pharmacia Biotech,
Uppsala, Sweden) were added to 40 μl of each PCR
product. Shrimp alkaline phosphatase was used to
15   degrade PPi and the dNTPs while exonuclease I removed
single-stranded DNA molecules including PCR primers.
The enzymatic degradation was allowed to proceed for 30
min at room temperature. The mixtures were then heated
to 97° C for 12 min to deactivate the enzymes. The
20   samples were divided into two tubes (20 μl in each) and
pairs of allele-specific primer (0.25 μM) (one
complementary to the forward strand and one
complementary to the reverse strand) were added into
each tube. The sequence of primers was A-forward
25   TACAACACTCCCTTCAGATCA, A-reverse TACCATTTGTTAAGCTTTTGT,
C-forward TACA ACACTCCCTTCAGATCC and C-reverse
ACCATTTGTTAAGCTTTTGG. The primer pairs in different
tubes differed only in their 3'-ends (underlined bases
indicate the alternating 3'-ends) to allow
30   discrimination by allele-specific extension using DNA
polymerase. After addition of primers, the samples were
incubated at 97° C for 2 min and then cooled to room
temperature, allowing hybridization of allele-specific
primer pairs. The content of each well was then further
35   divided into two separate reactions for comparison of
extension analysis with and without apyrase. Extension
and real-time luminometric monitoring was performed at

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25°C in a Luc96 pyrosequencer instrument
(Pyrosequencing, Uppsala, Sweden). An extension
reaction mixture was added to the samples (10 μl) with
annealed primers (the substrate) to a final volume of 50
5   μl. The extension reaction mixture contained 10 U
exonuclease-deficient (exo-) Klenow DNA polymerase
(Amersham Pharmacia Biotech, Uppsala, Sweden), 4 μg
purified luciferase/ml (BioThema, Dalarö, Sweden), 15 mU
recombinant ATP sulfurylase, 0.1 M Tris-acetate (pH
10   7.75), 0.5 mM EDTA, 5 mM Mg-acetate, 0.1% (w/v) bovine
serum albumin (BioThema), 1 mM dithiothreitol, 10 μM
adenosine 5'-phosphosulfate (APS), 0.4 mg
polyvinylpyrrolidone /ml (360 000), 100 μg D-
luciferin/ml (BioThema) and 8 mU of apyrase (Sigma
15   Chemical Co., St. Louis, MO, USA) when applicable.
Prior to nucleotide addition and measuring of emitted
light, pyrophosphate (PPi) was added to the reaction
mixture (0.08 μM). The PPi served as a positive control
for the reaction mixture as well as peak calibration.
20   All the four nucleotides (Amersham Pharmacia Biotech,
Uppsala, Sweden) were mixed and were dispensed to the
extension mixture (0.8 μM, final concentration). The
emitted light was detected in real-time.

25   **Results and Discussion**
       Figure 6 shows the results of bioluminometric
analysis of wiaf 1764. The SNP wiaf 1764 has the
variants G and/or T (C and/or A). Two pairs of allele-
specific primers were used to analyze this SNP, one
30   complementary to the forward strand and one
complementary to the reverse strand. The primer pairs
differed in their 3'-ends to allow discrimination by
allele-specific extension using DNA polymerase,
(extension 1 and extension 2 in Figure 6). Prior to
35   hybridization of allele-specific primers the PCR
products were treated by shrimp alkaline phosphatase and
exonuclease I to remove the excess of primers,

nucleotides and PPi. This allowed heat separation of
double-stranded DNA and direct analysis of the PCR
product without strand separation by using beads. Thus,
allele-specific extension was performed on both strands
5   of a double-stranded DNA. The bioluminimetric assay was
performed without using apyrase (- apyrase in Figure 6)
and by the use of apyrase (+ apyrase in Figure 6). All
three variants of wiaf 1764 were analyzed and the ratios
between the match and mismatch signals were calculated.
10   The ratios are outlined in the bottom of Figure 6. As
it is shown all variants of the SNP could correctly be
scored when apyrase was used in the system (ratios 4, 1
and 4.1 for samples g011, 119 and g055 respectively)
while two of the same samples were wrongly scored when
15   apyrase was not applied (ratios 1.5 and 1.2 for samples
g011 and g055 respectively).

        The advantage of using two primers in AMASE is that
these can be utilized in a PCR amplification assay when
a thermostable nucleotide-degrading enzyme (e.g.
20   alkaline phosphatase) is available. A perfectly match
primer pair will give rise to amplification while
mismatch primer pair will not. After amplification, the
PCR products are directly analyzed by a luminometric
assay since the same primer pair is used in allele-
25   specific extension. Another advantage of using two
allele-specific primers is that the sensitivity
increases by a factor of two because signals of two
extensions are obtained instead of one.

30   **Example 4**
          Introduced mismatch in allele-specific primers
                to improve allele-specific extension

        The present example describes a method of allele-
35   specific extension using apyrase and an introduced
mismatch in the allele-specific primer, with non-
stringent conditions for extension.

    PCR amplification of GPIIIa gene was performed as
described in the foregoing examples. The SNP in the
GPIIIa gene has the variants C/T (G/A). The PCR
products were immobilized on magnetic beads and single-

5   stranded DNA was obtained by alkaline treatment as
described in the foregoing examples. The immobilized
single-stranded DNA was used as target template in the
assay. Two different primers were designed. The
sequence of primers was CTG TCT TAC AGG CCC TGC CTG <u>C</u>G

10   for GPIIIC and CTG TCT TAC AGG CCC TGC CTG <u>T</u>G for
GPIIIT. The immobilized strand was resuspended in 32 μl
H$_2$O and 4 μl annealing buffer (100 mM Tris-acetate pH
7.75, 20 mM Mg-acetate). The single stranded DNA was
divided into two parallel reactions in a microtiter-

15   plate (18 μl/well) and 0.2 μM (final concentration) of
primers were added to the single stranded templates in a
total volume of 20 μl. The primers were designed so
that the 3'-end was one base after the SNP site and was
complementary to the target DNA (indicated in *italics*).

20   Thus, the base before the 3'-end (3'-1) was
complementary to the SNP variants and was the only
difference between the two allele-specific primers
(<u>underlined</u> bases). The base before the SNP site (3'-2)
on both primers (indicated in **bold**) was an introduced

25   mismatch to the target DNA (**G** on the primers and **G** on
the target DNA). Therefore, when the allele-specific
base in the primer does not match to the target
template, two mismatches (positions 3'-1 and 3'-2) and
one match (position 3') will be made between the

30   template and the last 3 bases in the primer (Fig. 7).
When the allele-specific base in the primer does match
to the target template, the last two bases in the 3'-end
of the primer (positions 3' and 3'-1) will be
complementary to the target DNA while the introduced

35   mismatch (position 3'-2) is not complementary. Raw-data
were obtained from a luminometric assay. The conditions
(enzymes, substrates etc) of performance of the

luminometric assay are as described in the foregoing
examples. Prior to nucleotide addition and measurement
of emitted light, pyrophosphate (PPi) was added to the
reaction mixture (0.02 μM). The PPi served as a
5   positive control for the reaction mixture as well as
peak calibration. All three variants of the SNP were
investigated with (+ apyrase) and without (- apyrase)
addition of apyrase (Fig. 7). As shown in Fig. 7,
Sample 1055 is homozygous A in the target DNA template.
10   Thus, an allele-specific primer containing the base C at
3'-1 position is a mismatch to the target (C to A
mismatch) and since the 3'-2 position is also a mismatch
(G to G mismatch), the two mismatches disrupt the
hydrogen bind of the match base at the 3' position of
15   the primer (G to C match) and no extension is observed.
When the allele-specific primer contains a matching base
to the target DNA at the 3'-1 position (T to A match),
two complementary bases are made (at 3'-1 and 3'
position) between the primer and template. In a non-
20   stringent extension condition (extension at 25° C), the
mismatch base at position 3'-2 does not remove the two
complementary bases at 3'-1 and 3', which leads to
extension. The same explanation can be used for sample
1267 that is homozygous G. In the heterozygous case
25   (sample 1001) both allele-specific bases are
complementary to the respective variant and give rise to
extension signals.

Example 5
Apyrase Mediated Allele Specific Extension
30   **on Microarray for Mutation Detection**

In this example, a microarray assay was used to
detect unknown mutations in a 15 base stretch in exon 5
35   of the p53 tumour suppressor gene. Partially
overlapping extension primers were designed, but instead
of two variants differing only in the 3'-end nucleotides

as for SNPs, all four possible variants of 3'-end were
used (Figure 8). This allowed characterization of all
possible mutations in a given position instead of two
allelic variants. Since four partially overlapping
5   primers are needed for detection and characterization of
mutations in a position, 60 oligonucleotides were
spotted on the chip for analysis of 15 bases. These
extension primers were then allowed to hybridize to
template, followed by extension *in situ* using
10   fluorescent nucleotides. After the extension the
microarray was scanned and fluorescent intensities were
measured and compared. Four different templates were
used: a 100% WT template and three mutated templates in
a 50% mixture with WT template.

15

Materials and Method

Oligonucleotides

The allele specific extension primers were
synthesized with a 5' amino group, which facilitates
20   covalent immobilization on the glass slide. A spacer
sequence of 15 T residues was included at the 5' end of
the gene specific sequence that contained 18 nucleotides
(Table 5). The gene specific sequences of the extension
primers were designed to have a Tm of 62-64^{0}C with the
25   3'-nucleotide hybridizing at the variant position. In
addition, amino linked oligonucleotides, which are
printed on the arrays to act as hybridization and
extension controls, were synthesized (Table 6). A
labeled hybridizing oligonucleotide, which was used for
30   normalization, was synthesized with a Cy3 label (Table
6). A 66-mer control extension target, which hybridizes
to the spotted extension control, was also synthesized
(nucleotides 274-341 of the HCV genome, accession number
M623321) (Table 6). Synthetic samples of 60-mers
35   (nucleotides 13205-19 of the p53 gene, accession number
U94788) were used to hybridize to the allele specific
extension primers (Table 7). These were either of wild-

type sequence or containing one mutation. The
oligonucleotides were synthesized by Thermo Hybaid,
Interactiva, (Germany) and MWG-Biotech, (Germany).

Table 5. Gene specific extension primers.

3'-Position	Sequence	3'-Position	Sequence
205T	(T)₁₅CGGAGGTTGTGAGGCGCT	212G	(T)₁₅TGTGAGGCGCTGCCCCCG
205A	(T)₁₅CGGAGGTTGTGAGGCGCA	212T	(T)₁₅TGTGAGGCGCTGCCCCCT
205C	(T)₁₅CGGAGGTTGTGAGGCGCC	213C	(T)₁₅GTGAGGCGCTGCCCCCAC
205G	(T)₁₅CGGAGGTTGTGAGGCGCG	213A	(T)₁₅GTGAGGCGCTGCCCCCAA
206G	(T)₁₅GGAGGTTGTGAGGCGCTG	213G	(T)₁₅GTGAGGCGCTGCCCCCAG
206A	(T)₁₅GGAGGTTGTGAGGCGCTA	213T	(T)₁₅GTGAGGCGCTGCCCCCAT
206C	(T)₁₅GGAGGTTGTGAGGCGCTC	214C	(T)₁₅TGAGGCGCTGCCCCCACC
206T	(T)₁₅GGAGGTTGTGAGGCGCTT	214A	(T)₁₅TGAGGCGCTGCCCCCACA
207C	(T)₁₅GAGGTTGTGAGGCGCTGC	214G	(T)₁₅TGAGGCGCTGCCCCCACG
207A	(T)₁₅GAGGTTGTGAGGCGCTGA	214T	(T)₁₅TGAGGCGCTGCCCCCACT
207G	(T)₁₅GAGGTTGTGAGGCGCTGG	215A	(T)₁₅GAGGCGCTGCCCCCACCA
207T	(T)₁₅GAGGTTGTGAGGCGCTGT	215C	(T)₁₅GAGGCGCTGCCCCCACCC
208C	(T)₁₅AGGTTGTGAGGCGCTGCC	215G	(T)₁₅GAGGCGCTGCCCCCACCG
208A	(T)₁₅AGGTTGTGAGGCGCTGCA	215T	(T)₁₅GAGGCGCTGCCCCCACCT
208G	(T)₁₅AGGTTGTGAGGCGCTGCG	216T	(T)I₅AGGCGCTGCCCCCACCAT
208T	(T)₁₅AGGTTGTGAGGCGCTGCT	216A	(T)₁₅AGGCGCTGCCCCCACCAA
209C	(T)₁₅GGTTGTGAGGCGCTGCCC	216C	(T)₁₅AGGCGCTGCCCCCACCAC
209A	(T)₁₅GGTTGTGAGGCGCTGCCA	216G	(T)₁₅AGGCGCTGCCCCCACCAG
209G	(T)₁₅GGTTGTGAGGCGCTGCCG	217G	(T)₁₅GGCGCTGCCCCCACCATG
209T	(T)₁₅GGTTGTGAGGCGCTGCCT	217A	(T)₁₅GGCGCTGCCCCCACCATA
210C	(T)₁₅GTTGTGAGGCGCTGCCCC	217C	(T)₁₅GGCGCTGCCCCCACCATC
210A	(T)₁₅GTTGTGAGGCGCTGCCCC	217T	(T)₁₅GGCGCTGCCCCCACCATT
210G	(T)₁₅GTTGTGAGGCGCTGCCCG	218A	(T)₁₅GCGCTGCCCCCACCATGA
210T	(T)₁₅GTTGTGAGGCGCTGCCCT	218G	(T)₁₅GCGCTGCCCCCACCATGC
211C	(T)₁₅TTGTGAGGCGCTGCCCCC	218C	(T)₁₅GCGCTGCCCCCACCATGG
211A	(T)₁₅TTGTGAGGCGCTGCCCCA	218T	(T)₁₅GCGCTGCCCCCACCATGT
211G	(T)₁₅TTGTGAGGCGCTGCCCCG	219G	(T)₁₅CGCTGCCCCCACCATGAG
211T	(T)₁₅TTGTGAGGCGCTGCCCCT	219A	(T)₁₅CGCTGCCCCCACCATGAA
212A	(T)₁₅TGTGAGGCGCTGCCCCCA	219C	(T)₁₅CGCTGCCCCCACCATGAC
212C	(T)₁₅TGTGAGGCGCTGCCCCCC	219T	(T)₁₅CGCTGCCCCCACCATGAT

hits/dot and with 2-4 replicate spots in each array.
Prior to spotting the extension primer solutions were
spiked with 2 μM of the 18-mer amino linked
hybridization control oligonucleotide, which hybridizes
to a labeled oligonucleotide with the duplex serving as
a hybridization and normalization control. The second
18-mer oligonucleotide, which acts as an extension
control, was also printed on the slide. This
oligonucleotide hybridizes to a 66-mer extension control
target oligonucleotide (mixed with the sample DNA) and
the duplex serves as a positive control for extension.
After printing, the arrays were incubated overnight in a
humid chamber followed by post coupling as outlined by
the manufacturer. Briefly the slides were incubated at
50^0C for 15 minutes in blocking solution (50 mM ethanol
amine, 0.1M Tris pH 9 and 0.1% SDS), rinsed twice in dH$_2$O
and washed with 4X SSC/0.1% SDS (prewarmed to 50^0C) for
15 to 60 minutes. The arrays were then rinsed in H$_2$O and
dried by centrifugation for 3 minutes at 800rpm.

Allele specific extension

4 pmol of synthetic single stranded 60-mers, either
100% wild-type or mixed equally to mutated
oligonucleotides (see Table 6), was incubated in 150 μl
5XSSC/0.2% SDS with 0.5 pmoles synthetic DNA (66 mer
extension control), 0.12 pmoles Cy3 labeled
hybridization control and 0.5 μg SSB (Amersham
Pharmacia, Biotech, Uppsala, Sweden) at 70^0C for 5
minutes. 140 μl of this solution was then hybridized to
the microarray in a GeneTAC hybridization station
(Genomic Solutions, MI, USA). Hybridization was
performed at 58^0C for 20 minutes followed by two washes
in 2X SSC/0.1% SDS (preheated to 58^0C) for 5 minutes
proceeded by washing in 0.2X SSC and 0.1X SSC for 1
minute. The slides were dried by centrifugation at 800
rpm for 1 minute prior to scanning with the Cy5 laser.
To perform extension, 100 μl of an extension mix

was prepared containing 80 mM Tris-Acetate, 0.4 mM EDTA,
1.4 mM MgAc$_2$, 0.5 mM DTT, 2 μg *E. coli* tRNA, 1 μg SSB, 20
units exonuclease-deficient (exo-) Klenow DNA polymerase
and 8 mU apyrase. Immediately prior to extension, 40 μl
5   of 2.5 μM dATP, dGTP, dTTP and Cy5 labeled dCTP
(Amersham Pharmacia BioTech) was added to the extension
mix and the solution injected over the slide. Extension
was performed for 15 minutes at 28^{0}C followed by a
washing procedure performed as described above.
10

Scanning and data analysis after extension
        The slides were scanned using the GMS 418 scanner
(Affymetrix, SA). Generally the optimal parameters were
laser and photomultiplier tube (PMT) settings of 100 %
15   and 50% respectively for Cy5 and laser and PMT settings
of 100% and 50% for Cy3. Sixteen-bit TIFF images were
then imported into GenePix 2.0 software (Axon
Instruments, USA) for analysis. After the median local
background was subtracted from the mean signal of the
20   spots, means for the 8 spots of each allele specific
extension primer printed on each slide were calculated.
The Cy3 channel signal was used as a visual aid as to
see if the hybridization were evenly distributed on the
chip.
25

Results and Discussions
        This example demonstrates the use of the micro
array assay to detect unknown mutations in a 15-base
stretch in exon 5 of the p53 tumour suppresser gene.
30   Partially overlapping extension primers were designed,
but instead of two variants differing only in the 3'-end
nucleotides as for SNPs, all four possible variants of
3'-end were used (Figure 8). This was to allow for all
three possible mutations instead of two allelic
35   variants. To perform detection and characterization of
mutations in these 15 bases, 60 partially overlapping
oligonucleotides were spotted on the chip. These

extension primers were then allowed to hybridise to a
synthetic 60-mer oligonucleotide template, followed by
extension in *situ* using fluorescent nucleotides. After
the extension the microarray was scanned and fluorescent
5   intensities were measured and compared (Figure 9). Four
different templates were used - 100% WT template and
three mutant templates in a 50% mixture with the WT
template (Table 7).

Averages of the intensities of the 8 replicates on
10   each slide are shown as diagrams in Figure 9. These
intensities were used to calculate the ratios between
the match signal and the mismatch signal.

This example demonstrates that unknown mutations
can be detected by apyrase mediated allele specific
15   extension.

Claims

1.   A method of detecting a base at a pre-determined position in a nucleic acid molecule, said method comprising:

     performing primer extension reactions using base-specific detection primers, each primer being specific for a particular base at said predetermined position, and comparing said primer extensions to determine which base is present at said position, wherein said primer extension reactions are performed using labelled nucleotides and wherein a nucleotide-degrading enzyme is present during the primer extension reaction.

2.   A method as claimed in claim 1, wherein one of said detection primers comprises a base which is complementary to the base it is desired to detect (the target base) and another detection primer is non-complementary to said target base.

3.   A method as claimed in claim 2, wherein said base in the primer corresponding to the target base is at the 3' end of the primer.

4.   A method as claimed in any one of claims 1 to 3 wherein the detection primers are allele-specific.

5.   A method as claimed in any one of claims 1 to 4 wherein two detection primers are used.

6.   A method as claimed in claim 5, wherein the first of said primers is specific for a non-mutated base at the predetermined position, and the second primer is specific for a mutated base at the predetermined position.

7.   A method as claimed in claim 5, wherein the first of said primers is specific for one allele, and the second primers is specific for a different allele.

8.   A method as claimed in any one of claims 1 to 4, for the detection of unknown base changes wherein four primers are used, each specific for one of the bases A, T, C or G.

9.   A method as claimed in claim 8, wherein each said base-specific detection primer comprises a different base (A, T, C or G) at its 3' terminus, or at any other "base-specific" position.

10.   A method as claimed in either one of claims 8 or 9, wherein a set of primers are used, each specific for a particular base at different pre-determined positions.

11.   A method as claimed in any one of claims 1 to 10, wherein the primer extension reactions are compared by determining and comparing the rates of extension, or extension efficiencies, of the respective primer extension reactions.

12.   A method as claimed in any one of claims 1 to 11 wherein the labelled nucleotide carries a fluorescent label.

13.   A method as claimed in any one of claims 1 to 12, wherein the label is attached to the nucleotide via a cleavable linker.

14.   A method as claimed in any one of claim 1 to 13 wherein the nucleotide degrading enzyme is apyrase.

15.   A method as claimed in any one of claims 1 to 14 wherein the target nucleic acid is amplified before

hybridization.

16.   A method as claimed in any one of claims 1 to 15,
wherein the target nucleic acid is immobilized.

5

17.   A method as claimed in any one of claims 1 to 14
wherein said method is performed in a solid phase
microarray format.

10   18.   A method as claimed in claim 17 wherein said
primers are immobilised.

19.   A method as claimed in any one of claims 1 to 18
wherein said primers are tagged on the 5'-ends by
15   barcodes.

20.   A method as claimed in any one of claims 1 to 19
wherein the target nucleic acid is double stranded.

20   21.   A method as claimed in any one of claims 1, 2 or 4
to 20, wherein the said base in the primer corresponding
to the target base is at the 3'-1 position in the primer
(i.e. the penultimate base of the primer).

25   22.   A method as claimed in claim 21, wherein the base
at the 3' position in said primer is complementary to
the base that is 5' to the predetermined position in the
target, and the base in the 3'-2 (ante-penultimate)
position in the primer is non-complementary to the base
30   that is 3' to the predetermined position in the target.

23.   A method for detecting an allele-specific base at a
predetermined position in a target nucleic acid
comprising conducting a first and a second allele-
35   specific primer extension reaction using a first and a
second primer, respectively that hybridizes to a region
of said target nucleic acid, each of said primers

having: 1) a 3'-end base that is complementary to the
base that is 5' of the predetermined position in the
target; 2) a base one position from the 3'-end that in
the first primer is complementary to a non-mutated based
at the predetermined position, and in the second primer
is complementary to a mutated base at the predetermined
position; and 3) a base two positions from the 3'-end
that is non-complementary to the base that is 3' of the
predetermined position in the target; and determining
primer extension efficiency in said first and second
reactions, whereby greater efficiency in said first
reaction is indicative of the presence of a non-mutated
base at said predetermined position, and whereby greater
efficiency in said second reaction is indicative of the
presence of a mutated base at said predetermined
position.

24.   The method of claim 23 wherein said extension
reactions are performed in the presence of a nucleotide
degrading enzyme.

25.   The method of claim 24 wherein said nucleotide
degrading enzyme is apyrase.

26.   The method of any one of claims 23 to 25 wherein
said target nucleic acid is amplified before
hybridization.

27.   The method of any one of claims 23 to 26 wherein
said primer extension efficiency is measured by an assay
selected from mass spectroscopy, a luminometric assay, a
fluorescent assay, and pyrosequencing.

28.   The method of any one of claims 23 to 27 wherein
said method is performed in a solid phase microarray
format.

29.   The method of any one of claims 23 to 28 wherein said primers are tagged at the 5'-ends by barcodes.

30.   The method of any one of claims 23 to 29 wherein said target nucleic acid is double stranded.
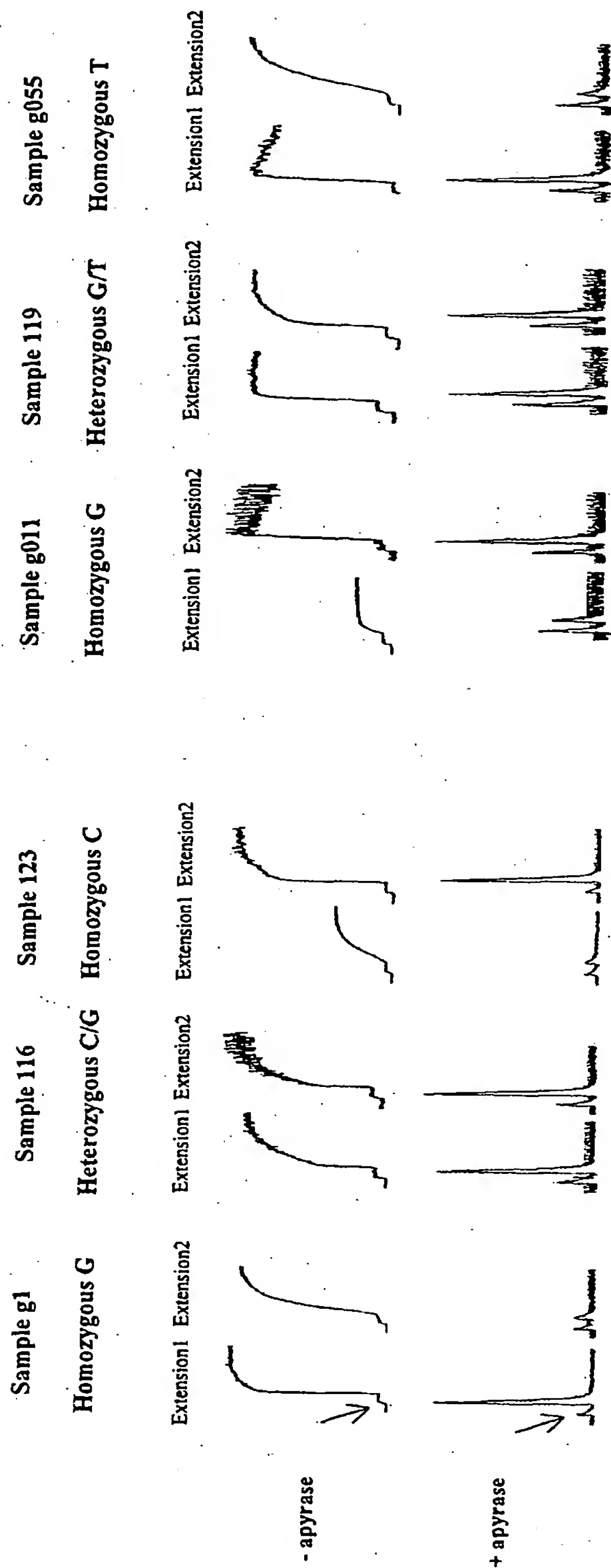
Fig 1 A

Fig 1 B
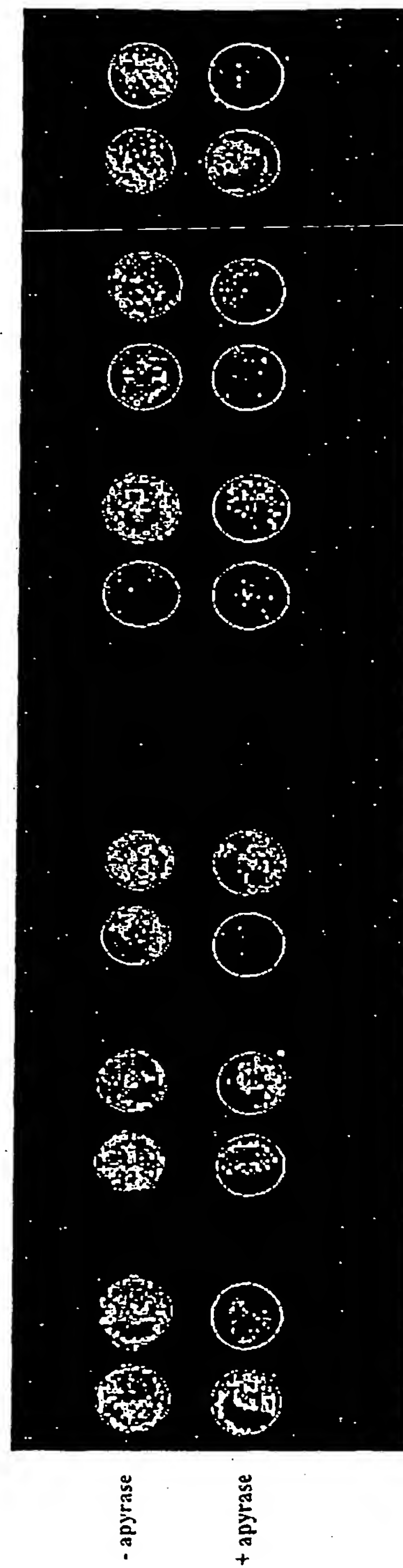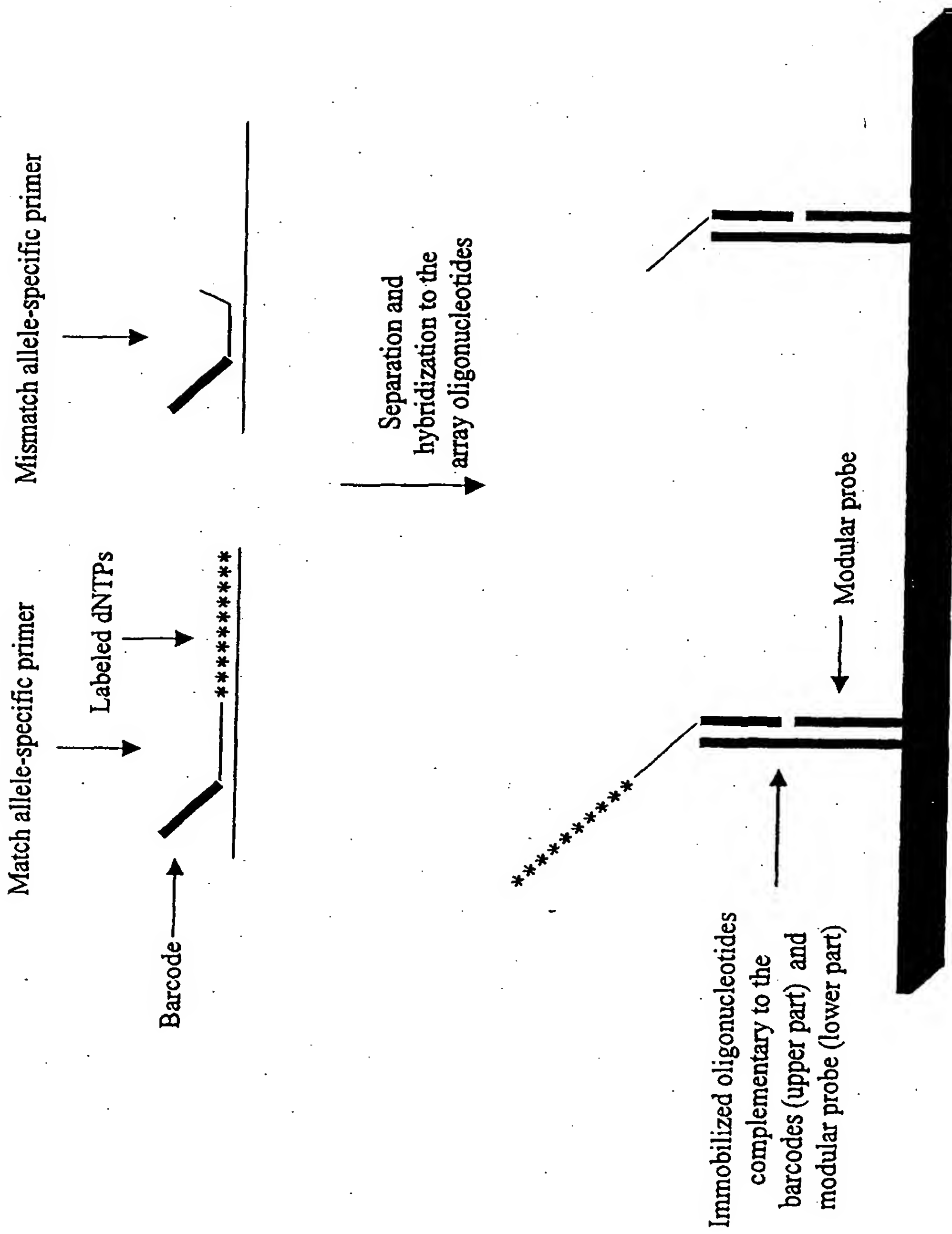
Fig 2
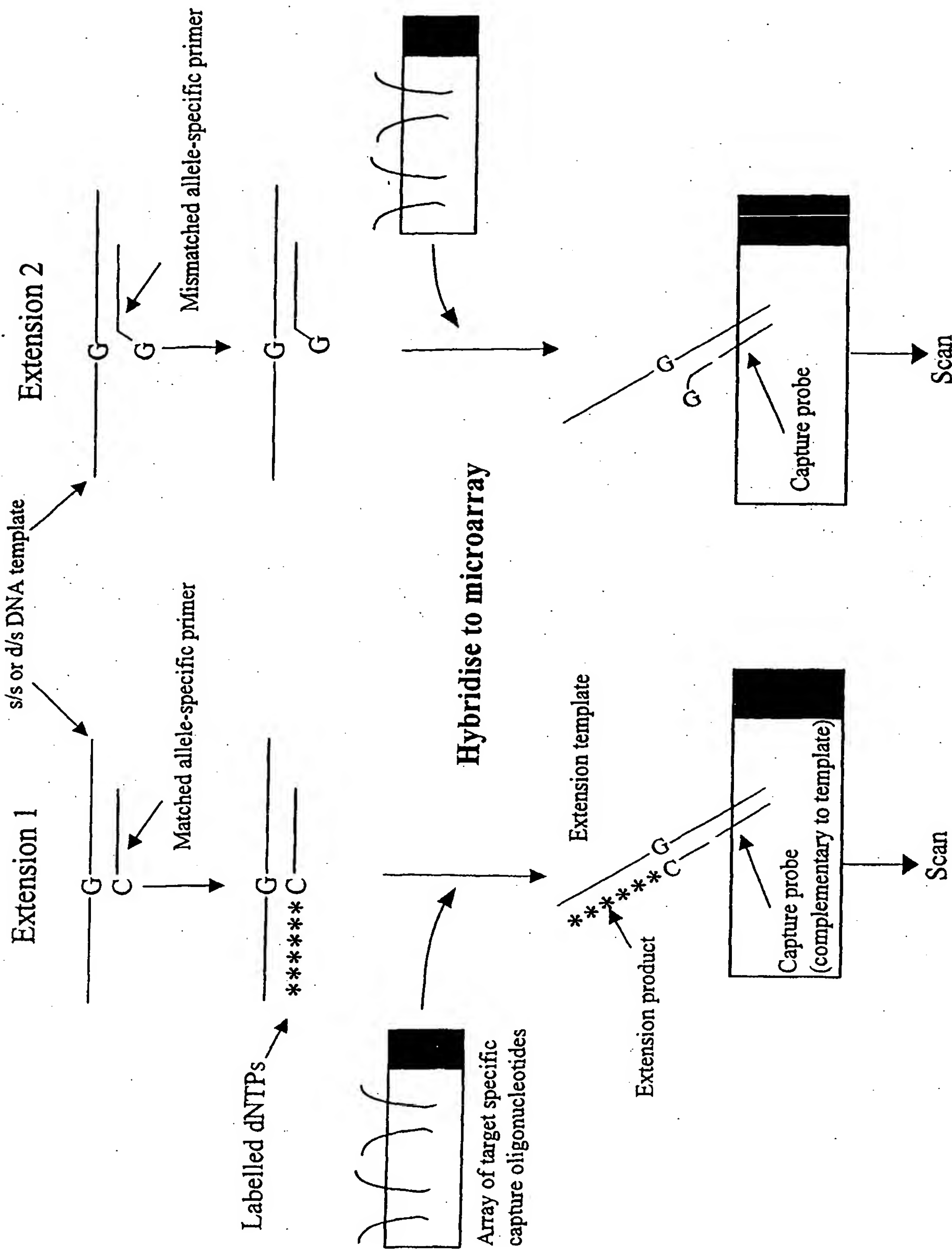
Fig 3

Exonuclease FRET (TaqMan) Assay by AMASE
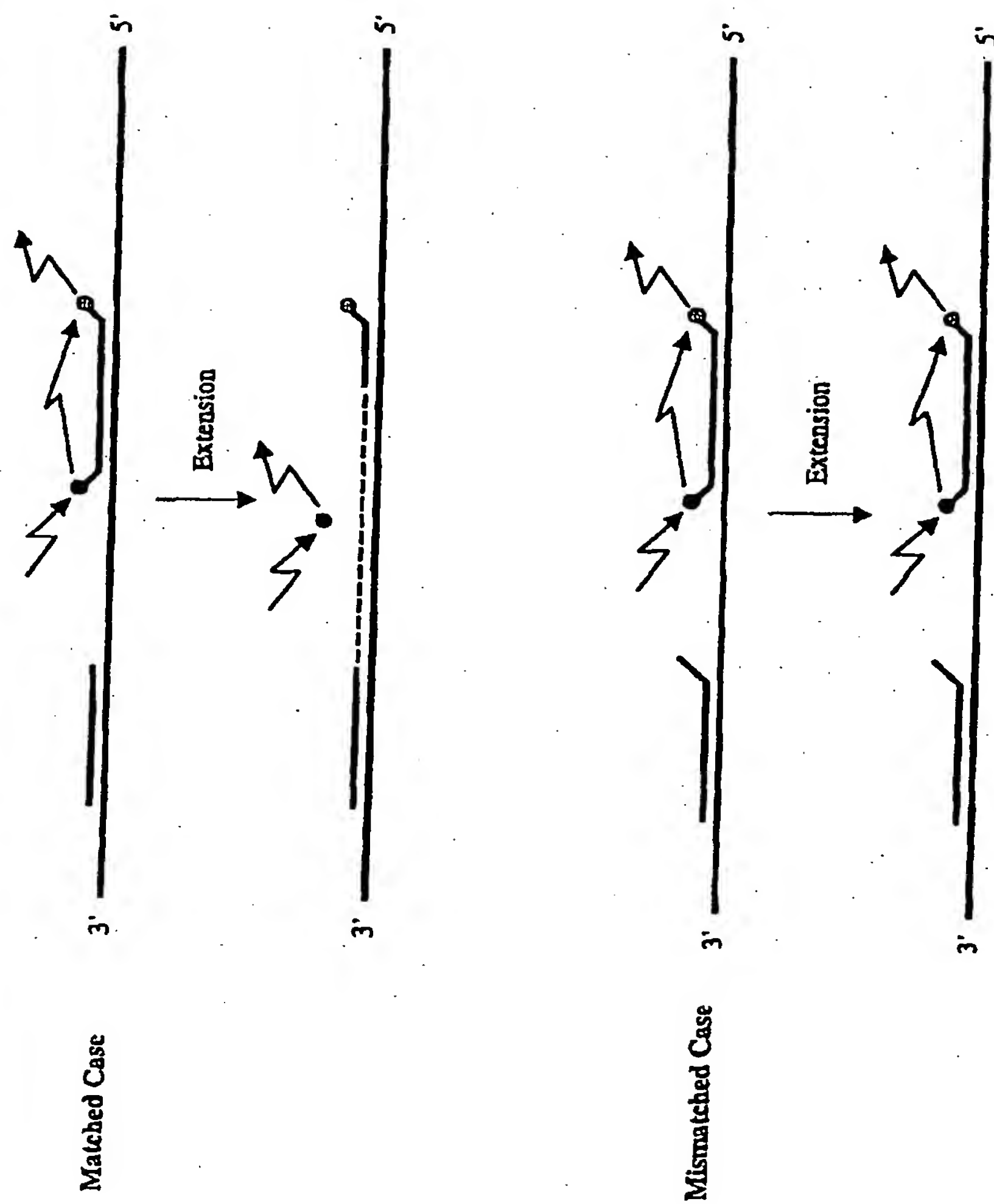
Fig 4
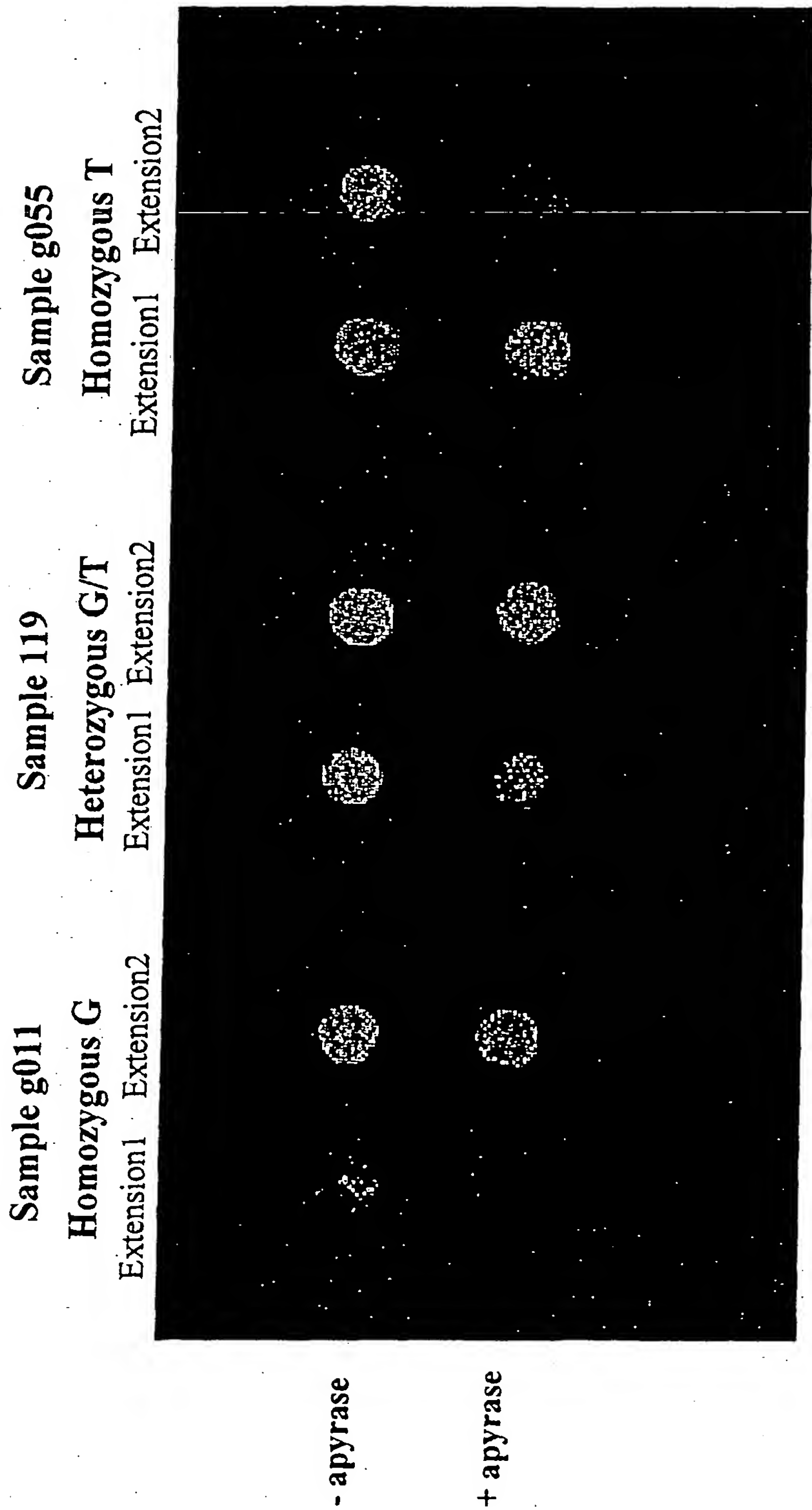
Fig 5

Bioluminometric analysis of wiaf 1764 by double allele-specific primer extension on double stranded DNA
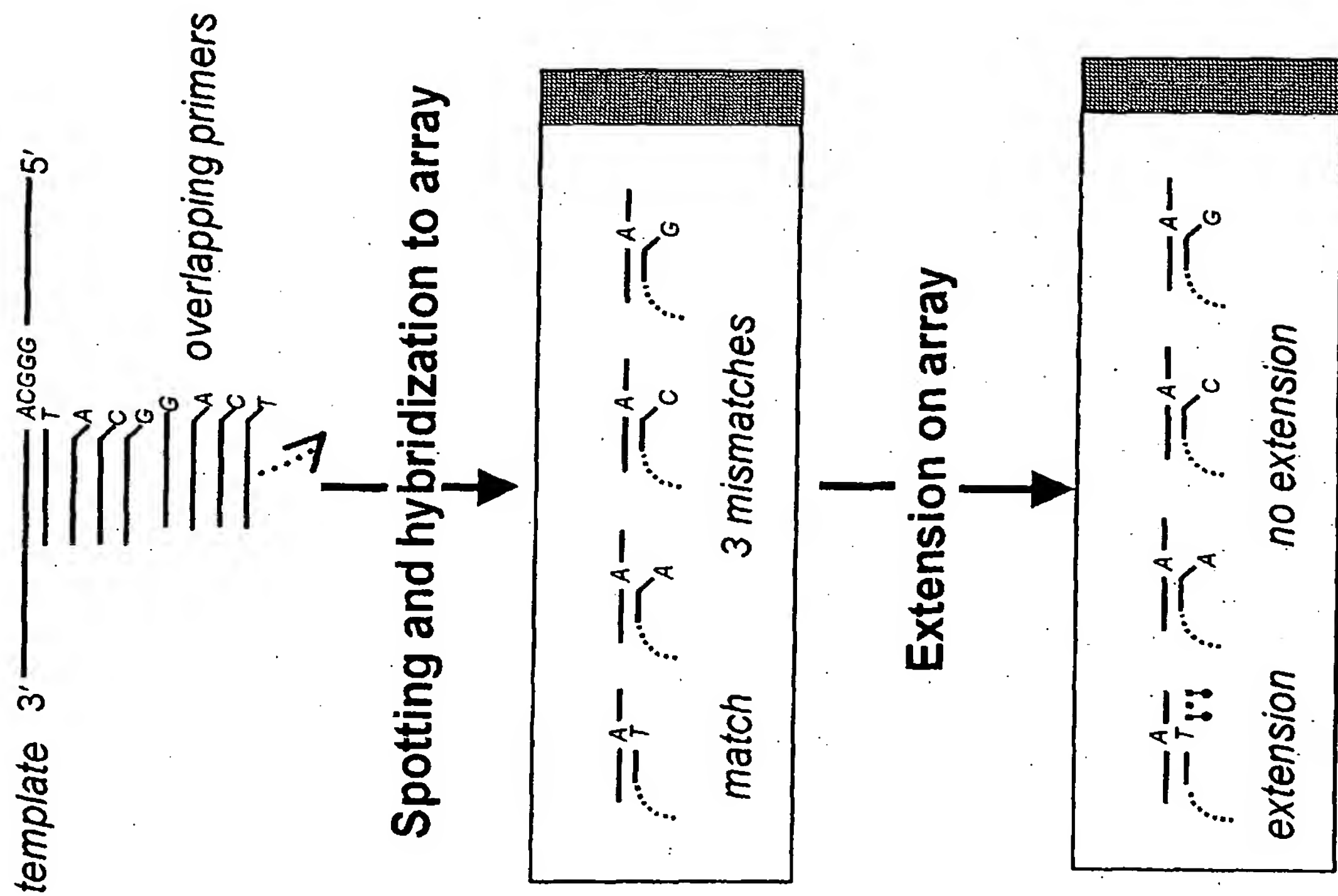
Fig 6

Fig 7

Fig 8

Fig 9